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ABSTRACT

African American men are at greater risk for developing and dying from prostate cancer compared to white men. This disparity is likely due to a number of factors including environmental and genetic factors. The Flint Men's Health Study (FMHS) was established in 1995 as a population-based case-control study of African American men aged 40-79 residing in Genesee County, Michigan. The initial sample consisted of 730 men who completed an in-home interview consisting of potential risk factors for prostate cancer; medical history; and demographic data. 431 men provided a blood sample and 369 men who were determined to be free of cancer completed a comprehensive urologic exam. Additionally, 119 cases of prostate cancer have been identified from the same population. Studies have suggested a role for hormones and genetics in cancer incidence. However, studies have been completed in white populations and results have been conflicting. The objective of this study is to more clearly delineate the potential role(s) of selected hormones and growth factors in prostate cancer development.

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INTRODUCTION

Prostate cancer is the most common cancer and the second leading cause of cancer deaths in U.S. men. African American men are at greater risk for both developing and dying from prostate cancer compared to white men. Research based on racial differences in prostate cancer has resulted in important discoveries that have just begun to unravel the complex biologic mechanisms for prostate cancer. The paucity of data on prostate cancer from population-based samples of African American men highlights a gap in our current understanding of the disease. The goal of this proposed study is to determine whether or not there are differences in 1) various circulating hormone and growth factor levels and 2) the prevalence of genetic polymorphisms between African American men with prostate cancer and those free of disease. We will also measure the associations between genetic polymorphisms and circulating hormone levels and their subsequent impact on prostate cancer risk in African American men. This information will enable us to better understand the role of hormonal and genetic risk factors in the disease process. We will make use of the unique opportunity presented by the availability of an ongoing epidemiologic study of community-dwelling African American men: the Flint Men's Health Study. We will accomplish this through the following Specific Aims:

Specific Aim 1: To evaluate the associations between *circulating hormone* and *growth factor* levels and *prostate cancer* using samples from a population-based cohort of African American men (Table 1).

Specific Aim 2: To evaluate the associations between *genetic polymorphisms* and *prostate cancer* using samples from a population-based cohort of African American men.
a. To evaluate differences in the prevalence of common *genetic polymorphisms* (Table 3) between men diagnosed with prostate cancer and disease free controls.
b. To evaluate differences in the prevalence of common *genetic polymorphisms* (Table 3) between men diagnosed with various stage and grade prostate cancer.

Specific Aim 3: To measure the associations between genetic polymorphisms and circulating hormone levels and their subsequent impact on prostate cancer risk in African American men.

The completion of these aims will lead to new insights into differences and/or similarities in the prevalence of hormonal and genetic correlates of prostate cancer between cases and controls. These insights will provide the direction for the next sets of studies to better define the etiology of prostate cancer in African American men, its natural history and clinic course, as well as potential targets for intervention.

BODY

The following include research accomplishments associated with tasks outlined in our approved Statement of Work.

Task 1. **To perform laboratory assays for the following hormones:**

Months 1-12
Total Testosterone
Free Testosterone
Androstenedione
Dihydroepiandrosterone sulfate
Serum hormone binding globulin
Androstenediol glucuronide
Sodium

Months 12-24
Estradiol
Estrone Sulfate
Insulin-like Growth Factor-1
Insulin-like Growth Factor Binding Protein-3

This task has been completed and included sending aliquots of over 450 serums to the appropriate laboratories, entering the data into spreadsheets, and data cleansing. Specifically, circulating hormone levels listed above were quantified by performing laboratory assays at the University of Michigan Medical Center Reproductive Sciences and Clinical Chemistry Labs. Total IGF-1, IGFBP-3 and AAG were measured by a commercially available enzyme-linked immunosorbent assays (ELISA) (Diagnostic Systems Laboratory, Webster, Texas) in Dr. Jaffe's laboratory. Inter-assay and intra-assay coefficients of variation were as follows: IGF-1: 4%,6%; IGFBP-3: 6%,9%; and AG: 5% and 11%. All other hormones were measured using commercially available chemiluminescent immunoassays (Bayer Diagnostics, Pittsburgh, PA) Inter-assay and intra-assay coefficients of variation, respectively, were as follows: DHEAS:18.14%, 12.53%; TT:8.68%, 6.82%; FT:6.5%, 7.3%; SHBG:18.95%, 10.31%; Androstenedione:11.6%, 6 %; E2:10.21%, 6.375%; and Estrone:10.27%, 10.93%.

Task 2. **To perform DNA analyses to examine the following genotypes:**

Months 1-12	Months 12-24
LHB	CYP19
HSD3B2	CYP3A4
CYP17	IGF1
HSD17B2	

We had initially planned to perform our SNP assays using either ABI PRISM® 7700 Sequence Detection System which uses TaqMan® assays or an ABI 3100 PRISM® Genetic Analyzer which employs a 16 capillary electrophoresis system. However the University of Michigan Comprehensive Cancer Center cDNA Core recently purchased the ABI PRISM® 7900 Sequence Detection System which has the capacity to perform high-throughput SNP detection using 384-well plates. We have performed a number of pilot experiments and have learned that SNP assays using the ABI 7900 are very sensitive to DNA concentration. We have optimized our assays and the failure rate is now less than 5%. This new genotyping platform has the reduced the estimated cost per genotype from $2.00 to less than $0.50. This allowed us to perform additional genotyping. Additionally in the face of new analytical methods for SNP selection and analyses we have revised our genotype list from the one listed above.

In summary, we completed in our lab genotyping for the PSA and Insulin genes using methods described above. Genotyping for the AR, CYP11A1, SRD5A2 genes were completed at the Mayo Clinic while assays for the MSR1 genotypes were completed at Wakeforest University. A manuscript describing data from the MSR1 analyses was published in *Cancer Research* (Appendix 1)[1] and PSA and Insulin genotyping findings have been submitted and are currently under review at *Cancer Epidemiology, Biomarkers and Prevention* and the *Prostate* journals, respectively (Appendix 1). Data quality assessment, cleansing and analyses of AR, CYP11A1 and SRD5A2 are currently in progress. We have decided to outsource our genotyping for the remainder of the SNPs in the interest of time and money. The following SNPs have been selected and are being prepared for assay completion by Fall 2005:

IGFBP-3	rs3110697	**CY3A4**	rs12333983
	rs3793345		rs2242480
CYP17	rs10883783		rs2740574
	rs6163	**CYP19**	rs7000519
	rs6162	**IGF-1**	CA repeat
	rs743572		

Task 3. **Interim statistical analyses of data obtained from hormone assays and DNA will be performed periodically as assays are performed. (Months 1-24)**
For years 1-2, we completed preliminary statistical analyses of proposed serum hormone data and statistical analyses of genotyping have been completed for the following genes: MSR1, PSA and Insulin. Analyses of AR, CYP11A1 and SRD5A2 data are currently in progress. Additionally, more comprehensive analyses and manuscript preparation for various serum hormone data will being following analyses of control serum temporality and batch variation currently in progress.

Task 4. **Final analyses and report writing (Months 24-36)**
a. Final analyses of data from hormone assays and genetic polymorphisms will be performed.
b. Final manuscripts will be prepared and submitted.

One manuscript has been published and two additional manuscripts have been completed and are currently under review. Final analyses of hormone data are currently in progress and analyses and report writing of remaining genetic polymorphisms will be completed in the final year.

KEY RESEARCH ACCOMPLISHMENTS

- A significant difference in median values of sex hormones was observed between serum from African American prostate cancer cases and disease-free controls
- Selected rare macrophage scavenger receptor 1 gene mutations were observed to be associated with increase prostate cancer susceptibility among African American men
- Polymorphisms in the prostate specific antigen gene promoter did not predict serum prostate specific antigen levels nor prostate cancer risk in African American men
- Insulin gene *PstI* CC genotype was positively associated with prostate cancer diagnosis and lower grade and stage of prostate cancer in African-American men. INS *PstI* genotype was not associated with later age of diagnosis.

REPORTABLE OUTCOMES

In this first 2 years of funding, we have made significant progress towards completing laboratory studies required to carry out analyses described in Specific Aims 1, 2 and 3. Table 1 reports initial comparisons of mean serum hormone concentrations by prostate cancer status. Control serums are currently being evaluated for temporal and batch variations. Once evaluations are complete, additional analyses will be performed to examine potential confounders in the relationship between these circulating hormones and the risk of prostate cancer and to determine whether significant differences in serum hormone concentrations exist by stage and grade of disease.

Table1 . Median (25%, 75%) values of sex hormone-related factors according to prostate cancer status

Hormone	Prostate Cancer (n=124)	Disease-free controls (n=406)	P-value*
Androstenedione (ng/ml)	6.28 (3.90, 9.06)	1.00 (0.80, 1.30)	**<0.0001**
Estradiol (pg/ml)	36.70 (26.75, 43.5)	28.90 (22.90, 36.40)	**<0.0001**
Estrone (ng/ml)	1.44 (1.04, 1.87)	1.91 (1.25, 2.94)	**<0.0001**
SHBG (nM)	42.15 (30.20, 56.60)	29.70 (21.50, 43.10)	**<0.0001**
Total testosterone (ng/dl)	445.50 (321.46, 611.62)	559.77 (413.88, 757.10)	**<0.0001**
IGF-1 (ng/ml)	233.66 (183.55, 288.16)	59.00 (41.00, 83.00)	**<0.0001**
IGFBP-1(ng/ml)	39.62 (31.89, 46.52)	3.32 (2.60, 4.33)	**<0.0001**

* Kruskal-Wallis Test.

In addition, several manuscripts have been completed (1 published and 2 currently under review) examining the association between various SNPs and risk of prostate cancer in African-American men. Below are the published/submitted abstracts and presentation of data for the three completed analyses.

"Germ-line Mutations of the Macrophage Scavenger Receptor 1 Gene: Association with Prostate Cancer Risk in African American Men." *Cancer Research. 63, 3486-89. 2003.*

Both rare germ-line mutations and common sequence variants of the macrophage scavenger receptor 1 (*MSR1*) gene have recently been implicated as potential prostate cancer susceptibility factors. However, existing studies are limited by the referral-based nature of samples and a paucity of African-American participants. In this context, we evaluated the association of germ-line mutations and common *MSR1* sequence variants with prostate cancer risk in a case control study of a community-based sample of 134 African-American men with prostate cancer and 340 unaffected controls. In our sample, the rare Asp174Tyr missense change was identified nearly twice as frequently in men with prostate cancer (6.8%) compared with unaffected controls (3.6%; $P = 0.14$). Moreover, significantly different allele frequencies between cases and controls were observed for one of the sequence variants, IVS5–59 ($P = 0.02$). Taken together, our results provide some additional support for the hypothesis that selected, rare *MSR1* mutations are associated with increased prostate cancer susceptibility among African-American men. (see Appendix 1)

"Polymorphisms in the Prostate Specific Antigen Gene Promoter Do Not Predict Serum Prostate Specific Antigen Levels in African American Men." currently under review at *Cancer Epidemiology, Biomarkers and Prevention.*

A major problem with the use of serum PSA in predicting prostate cancer risk is the considerable intra-individual variability of such measurements. Cramer et al. (2003) identified a set of single nucleotide polymorphisms (SNPs) in the upstream regulatory region of the PSA gene that were each associated with increased promoter activity and serum PSA, further suggesting that genotyping these SNPs could be useful in improving the predictive value of PSA screening. In order to replicate this finding, DNA samples from 475 African American men were genotyped for the same SNPs and no association was observed with either serum PSA level or prostate cancer diagnosis. Specific data are presented in Tables 2-5 below.

Table 2. Frequency of three SNPs in the PSA gene promoter region among African American men

›n of SNP	Nucleotide Change	% control chromosomes carrying variant (n = 2 x 339)	% case chromosomes carrying variant (n = 2 x 136)
-4643:rs925013 *(NcoI)*	A to G	9.3 (63/678)	8.8 (24/272)
-5412:rs2739448*(BstUI)*	T to C	9.3 (63/678)	8.8 (24/272)
-5429:rs2569733 *(FokI)*	T to G	9.9 (67/678)	8.5 (23/272)

Table 3. Frequencies of selected SNPs in the promoter region of the PSA gene among 136 men with prostate cancer and 339 healthy control men participating in the FMHS

SNP/Genotype	Prostate Cancer Cases N (%)	Controls* N (%)	OR† (95%CI)
-4643:rs925013 *(NcoI)*			
AA	113 (83.1)	277 (81.7)	
AG	22 (16.2)	61 (18.0)	
GG	1 (0.7)	1 (0.3)	0.91 (0.54-1.55)
-5412:rs2739448 *(BstUI)*			
TT	113 (83.1)	277 (81.7)	
TC	22 (16.2)	61 (18.0)	
CC	1 (0.7)	1 (0.3)	0.54 (0.47-1.55)
-5429:rs256733 *(FokI)*			
TT	114 (83.8)	273 (80.5)	
TG	21 (15.4)	65 (19.2)	
GG	1(0.7)	1 (0.3)	0.80 (0.47-1.36)

* Controls with a serum PSA level of greater than or equal to 9.0 ng/mL were excluded.
† Odds ratio comparing individuals with at least one copy of the rare allele (heterozygotes or homozygotes) compared to individuals who were homozygotes for the more prevalent allele.
‡ Age-adjusted comparison between men who are heterozygotes or homozygotes for the least prevalent allele compared to men who are homozygotes for the most prevalent allele.

Table 4.Least square means, adjusted for age, of log10 total, log10 free and the ratio of free to total PSA by genotype in PSA promoter region SNPs among 339 control participants of FMHS*SNP/Genotype†

	N (%)	Mean Log (Total PSA ng/mL) (SD)	P-value‡	Mean Log (Free PSA ng/mL) (SD)	P-value
-4643:rs925013 *(NcoI)*					
AA	277 (81.7)	-0.039 (0.394)		-0.637 (0.345)	
AG	61 (18.0)	-0.048 (0.289)		-0.650 (0.230)	
GG	1 (0.3)	0.613 (N/A)	0.88	-0.268 (N/A)	0.98
-5412:rs2739448 *(BstUI)*					
TT	277 (81.7)	-0.040 (0.394)		-0.636 (0.344)	
TC	61 (18.0)	-0.046 (0.0.294)		-0.652 (0.301)	
CC	1 (0.3)	0.613 (N/A)	0.86	-0.268 (N/A)	0.90
-5429:rs256733 *(FokI)*					
TT	273 (80.5)	-0.037 (0.393)		-0.635 (0.345)	
TG	65 (19.2)	-0.057 (0.305)		-0.655 (0.303)	
GG	1 (0.3)	0.613 (N/A)	0.89	-0.268 (N/A)	0.80

* Men with a serum PSA level of greater than or equal to 9.0 ng/mL were excluded.
† Nucleotide position relative to the transcription start site.

Table 5.Association between PSA promoter haplotypes and log10 total serum and free PSA levels in 339 controls

Haplotype	Allele -5429 T/G SNP	-5412 T/C SNP	-4643 A/G SNP	Estimated Frequency	Log10 Total PSA Score†	P*	Log10 Free PSA Score†	P*
1	T	T	A	0.900	-.21	0.83	0.11	0.91
2	G	C	G	0.091	.37	0.71	-0.10	0.92
3**	G	C	A	0.003	NA	NA	NA	NA
4**	G	T	A	0.003	NA	NA	NA	NA
5**	G	T	G	0.003	NA	NA	NA	NA
Global					0.34	0.84	0.01	0.99

† HAPLO.SCORE (http://www.mayo.edu/statgen) program statistic.
* Chi-square test of the score test statistic. PSA level adjusted for age.
** Given rarity of haplotype, haplotype not evaluated in statistical tests of association.
A significant p-value and a positive (negative) score for a particular haplotype would have suggested that the haplotype was associated with increased (decreased) levels of the PSA outcome variable.

"INS PstI Polymorphism and Prostate Cancer Risk in African-American Men: The Flint Men's Health Study" currently under review at *Prostate*

African-American men have the highest rate of prostate cancer, and have also been found to have higher levels of serum insulin. It is hypothesized that one of the causes for the higher rate of prostate cancer in African-American men is due to an increased prevalence of the CC genotype of the INS *PstI* polymorphism in the 3'UTR of the INS gene. This genotype is hypothesized to lead to higher levels of serum insulin that subsequently increases the risk of prostate cancer diagnosis. We examined the association between INS PstI genotype and prostate cancer diagnosis in a population-based case-control study of African-American men aged 40-79 using data from the Flint Men's Health Study.

In 1996, 369 African-American men free of prostate cancer, completed a detailed in-home epidemiologic interview, completed a prostate cancer screening protocol, and had anthropometric measurements and a blood sample taken. 129 African-American men, aged 40-79, residents of Genesee County, and diagnosed with prostate cancer between 1996-2001 were identified using the local cancer registry and completed the interview, had anthropometric measurements and a blood sample taken, and had their hospital and registry records reviewed pertaining to their diagnosis of prostate cancer. Genotype data was collected and analyzed on 124 cases and 342 controls by restriction digests and a sequencing machine. The INS *PstI* genotype was analyzed for its association with prostate cancer diagnosis and severity, and for its association with age of diagnosis of prostate cancer, using multiple variable logistic regression. Other factors including age, BMI, diabetes, and family history were considered in the analyses.

We observed that men with the INS *PstI* CC genotype were more likely to have prostate cancer compared with the TT/TC genotypes (OR=1.63; 95% CI=0.91, 2.91), although this association was not statistically significant (P=0.09). INS *PstI* CC genotype was more highly associated with prostate cancer among men who reported a history of diabetes (OR=2.52, 95% CI 0.84, 7.56). The adjusted odds of INS *PstI* CC genotype was higher for those with T2 cancer (OR=1.50, 95% CI 0.52, 4.28) than for those with T3 cancer (OR=0.96, 95% CI 0.80, 11.54). The adjusted odds of having a higher grade of prostate cancer (Gleason grade =7) are lower for those with the INS *PstI* CC genotype compared to the TT or TC genotype (OR=0.55, 95% CI 0.20-1.55). The CC genotype was associated with a 2.53-fold greater odds of being diagnosed in the 60-69 age category as compared to the 50-59 age category (95% CI 0.67-9.53), suggesting that the CC genotype is associated with a later age of diagnosis. However, the

adjusted odds ratio was 1.07 times greater for the CC genotype for being diagnosed in the 70-79 age category compared to the 50-59 age category (95% CI 0.31-3.62), which contradicts this finding.

We observed that INS *PstI* CC genotype was positively associated with prostate cancer diagnosis in this population-based cohort of African-American men aged 40-79 after adjusting for age, BMI, diabetes, and family history. Furthermore, the INS *PstI* genotype was associated with a lower grade and stage of prostate cancer, although these associations were not statistically significant. The INS *PstI* genotype was not associated with a later age of diagnosis as was previously reported. Future longitudinal studies may be necessary that take into account serum insulin levels and that utilize a larger sample size. Specific data are presented in Tables 6-12 below.

Table 6: Sample characteristics by case/control status and odds ratios with 95% confidence intervals for prostate cancer diagnosis

	Cases (n=124)	Controls (n=342)	Crude Odds Ratio (95% CI)	
	N (%)	N (%)		$?^2$ trend
Age				**p<.0001**
70-79	37 (29.84%)	43 (12.57%)	22.80 (7.66, 67.87)	
60-69	51 (41.13%)	86 (25.15%)	15.72 (5.46, 45.21)	
50-59	32 (25.81%)	107 (31.29%)	7.93 (2.71, 23.19)	
40-49	4 (3.23%)	106 (30.99%)	--	
BMI				p=.14
Obese (>30)	38 (31.15%)	95 (27.78%)	1.49 (0.87, 2.55)	
Overweight(25-29.9)	51 (41.80%)	124 (36.26%)	1.53 (0.93, 2.54)	
Normal (<25)	33 (27.05%)	123 (35.96%)	--	
Family history				--
Yes	27 (21.77%)	72 (21.05%)	1.04 (0.63, 1.72)	
No	97 (78.23%)	270 (78.95%)	--	
Diabetes				--
Yes	29 (23.39%)	61 (17.84%)	1.41 (0.85, 2.32)	
No	95 (76.61%)	281 (82.16%)	--	

**Row totals may not add up to the total N due to missing data.

Table 7: Frequency of INS PstI genotype by case/control status and odds ratios with 95% confidence intervals for genotype and prostate cancer diagnosis (adjusted for age, BMI, diabetes, and family history)

	Cases (n=124)	Controls (n=342)	Crude OR (95% CI)*
CC	104 (83.87%)	262 (76.61%)	1.59 (0.93, 2.72)
TC	19 (15.32%)	78 (22.81%)	--
TT	1 (0.8%)	2 (0.6%)	

*Odds ratio compares CC genotype with the TC and TT genotypes combined, due to the small TT sample size (n=3).

Table 8: Stratified odds ratios with 95% confidence intervals of INS *PstI* genotype and prostate cancer diagnosis			
	N	Crude OR (95% CI)	P-value **
Diabetes			
Yes	90	2.52 (0.84, 7.56)	P=0.37
No	376	1.42 (0.76, 2.64)	
BMI			
Obese (=30)	133	1.61 (0.60, 4.34)	
Overweight (25-29.9)	175	1.49 (0.65, 3.41)	P=0.99
Normal (=25)	156	1.65 (0.58, 4.67)	
Family History			
Yes	99	1.44 (0.51, 4.08)	P=0.82
No	367	1.66 (0.88, 3.13)	
Age			
70-79	80	1.75 (0.64, 4.80)	
60-69	137	2.61 (0.91, 7.49)	P=0.35
50-59	139	0.87 (0.33, 2.29)	
40-49	110	*	

*Odds ratio not calculated due to all cases in 40-49 age category (n=4) having the CC genotype.
** Breslow-Day test of homogeneity.

Table 9: Multivariable odds ratios with 95% confidence intervals of INS PstI genotype and prostate cancer risk*	
	Multivariable Odds Ratio (95% CI)
INS *PstI* Genotype	
CC	1.63 (0.91, 2.91)
TT or TC	--
BMI	
Obese	**1.86 (1.03, 3.38)**
Overweight	1.43 (0.84, 2.45)
Normal	--
Diabetes	
Yes	0.90 (0.51, 1.56)
No	--
Family History	
Yes	1.12 (0.64, 1.96)
No	--
Age	
70-79	**34.45 (9.90, 119.91)**
60-69	**21.97 (6.55, 73.63)**
50-59	**11.16 (3.30, 37.76)**
40-49	--

* Variables in model adjusted for all other variables included above.

Table 10: Stage of prostate cancer cases, genotype frequencies, and multivariable odds ratios of INS *PstI* genotype and stage of cancer with 95% confidence intervals (adjusted for age, BMI, diabetes, and family history)

Stage	N (%)	% CC Genotype	Adjusted OR*	Adjusted OR comparing T3 (regionalized) vs. T1 and T2 (localized)
T3	4 (3.51%)	3 (75.00%)	0.96 (0.80, 11.54)	
T2	55 (48.25%)	47 (85.45%)	1.50 (0.52, 4.28)	0.76 (0.07, 8.09)
T1	55 (48.25%)	44 (80.00%)	--	

*Chi square trend=.6455

Table 11: Gleason grade of prostate cancer cases, genotype frequencies, and multiple variable odds ratios of INS *PstI* genotype and severity of cancer with 95% confidence intervals (adjusted for age, BMI, diabetes, and family history)

Gleason grade	N (%)	% CC Genotype	Adjusted OR
= 7	63 (53.39%)	50 (79.37%)	0.55 (0.20, 1.55)
= 6	55 (46.61%)	48 (87.27%)	--

Table 12: Frequency of age of diagnosis of cases, genotypes and multivariable odds ratios with 95% confidence intervals of INS *PstI* genotype and age of diagnosis (adjusted for BMI, diabetes, and family history)

Age at Diagnosis	N (%)	% CC Genotype	Adjusted OR
70-79	37 (29.84%)	29 (78.38%)	1.07 (0.31, 3.62)
60-69	51 (41.13%)	46 (90.20%)	2.53 (0.67, 9.53)
50-59	32 (25.81%)	25 (78.13%)	*
40-49	4 (3.23%)	4 (100.00%)	--

*50-59 was used as the reference group since the 40-49 group is all CC genotype.

CONCLUSIONS

Given the overall increased incidence of and mortality due to prostate cancer in African-American men, these men represent an appropriate population for the study of the associations between circulating hormones and genetic polymorphisms and prostate cancer risk. Our goal is to identify hormonal factors and genetic markers that can be used to stratify African-American men who are at risk for developing disease as well as those who progress to more severe disease. Over the next 12 months, we will complete our analyses of the hormone and genotype data with respect to prostate cancer risk. We will complete our genotyping of selected SNPs in candidate susceptibility genes to test for association with prostate cancer using the FMHS population.

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[CANCER RESEARCH 63, 3486–3489, July 1, 2003]

Advances in Brief

Germ-line Mutations of the Macrophage Scavenger Receptor 1 Gene: Association with Prostate Cancer Risk in African-American Men[1]

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Abstract

Both rare germ-line mutations and common sequence variants of the macrophage scavenger receptor 1 (*MSR1*) gene have recently been implicated as potential prostate cancer susceptibility factors. However, existing studies are limited by the referral-based nature of samples and a paucity of African-American participants. In this context, we evaluated the association of germ-line mutations and common *MSR1* sequence variants with prostate cancer risk in a case control study of a community-based sample of 134 African-American men with prostate cancer and 340 unaffected controls. In our sample, the rare Asp174Tyr missense change was identified nearly twice as frequently in men with prostate cancer (6.8%) compared with unaffected controls (3.6%; $P = 0.14$). Moreover, significantly different allele frequencies between cases and controls were observed for one of the sequence variants, IVS5–59 ($P = 0.02$). Taken together, our results provide some additional support for the hypothesis that selected, rare *MSR1* mutations are associated with increased prostate cancer susceptibility among African-American men.

Introduction

There is a growing body of molecular and genetic epidemiological evidence that implicates the short arm of chromosome 8 (8p22–23) as the location of one or more genes important in the development of adenocarcinoma of the prostate (1, 2). Most recently, the *MSR1*[3] gene has been proposed as an etiologic link between germ-line alterations in 8p and prostate carcinogenesis (3, 4). Xu *et al.* identified several rare germ-line mutations of the *MSR1* gene that cosegregated with prostate cancer among families affected with HPC. Moreover, at least one of the germ-line mutations was associated with an increased risk of sporadic prostate cancer among African-American men (4). In a subsequent study of men of European descent, the same authors examined five common sequence variants of *MSR1* and reported significantly different allele frequencies for each of the five variants among men diagnosed with prostate cancer compared with unaffected controls. Notably, the association of the common sequence variants with prostate cancer risk was independent of the presence of rare germ-line mutations (3).

The composite results of these studies provide provocative data in support of *MSR1* as a prostate cancer susceptibility gene. However, the generalizability of these findings is limited by a lack of African-Americans participants. Given that African-American men have both a higher incidence and mortality from prostate cancer compared with Caucasian men in the United States, characterization of genetic risk factors in this patient population is an important public health initiative, and further study of a potential role for *MSR1* is warranted (5). The aim of this study is to further evaluate the association between genetic variation in the *MSR1* gene and prostate cancer susceptibility among African-American men.

Materials and Methods

Subjects. Both cases and controls were recruited as part of the FMHS. Informed consent was obtained from each study participant, and all research protocols were approved by the Institutional Review Board at the University of Michigan Medical School. As described previously, disease-free controls, aged 40–79, were identified from a probability sample of African-American men in the city of Flint, Michigan or in neighboring Beecher Township (Genesee County; Ref. 6). A complete urological history and physical examination, including PSA testing, was performed to exclude the diagnosis of prostate cancer. Participating community urologists used the PSA values in conjunction with other clinical data to determine the need for biopsy; in general, a PSA value of >4 ng/ml indicated the need for biopsy. DNA was available for genetic sequencing for 345 unaffected men; however, the DNA was insufficient for 5 individuals. Thus, our final control sample consists of 340 disease-free African-American males.

Prostate cancer case recruitment from the same community was initiated in 1999 and completed in July 2002. Participation of cases required: (*a*) an epidemiological interview; (*b*) a review of the hospital and registry records for information on tumor stage, Gleason Score, prediagnosis PSA, and type of therapy; and (*c*) provision of a blood sample for DNA and freezer storage of serum and plasma. After excluding two cases with insufficient DNA, our final case sample included 134 African-American men, aged 40–79, that had been diagnosed with prostate cancer between 1995 and 2002. For both cases and controls, genomic DNA was isolated from whole blood by the use of the Puregene kit (Gentra Systems, Inc., Plymouth, MN).

Sequence Analysis. Five common sequence variants and five recently reported rare germ-line mutations were analyzed for 134 cases and 340 unaffected controls. The five rare mutations were identified during screening for sequence variants of *MSR1* in germ-line DNA samples from individuals with HPC (4). Four are missense mutations (Ser41Tyr, Asp174Tyr, Gly294Glu, and Pro36Ala), and one is a nonsense change (Arg293X). The five common sequence variants genotyped have been described previously and include an SNP in the promoter sequence (PRO3), a 15-bp insertion/deletion variant in intron 1 (INDEL1), an SNP located in intron 5 (IVS5-59), a missense mutation in exon 6 (P275A), and a 3-bp insertion/deletion in intron 7 (INDEL7; Ref. 3). The method of identification and positions of the five sequence variants have been reported elsewhere (3).

Statistical Analysis. Bivariate comparisons of mutation and allele frequencies among cases and controls were carried with χ^2 analysis or Fisher's exact test. Logistic regression models were used to test the association between common variants and disease status. These models were age adjusted to account for the possibility that some of the controls may later become diagnosed as cases. To avoid bias, age was calculated based on the same date for all cases and controls. This date was the most recent follow-up date from the

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[3] The abbreviations used are: *MSR1*, macrophage scavenger receptor 1; HPC, hereditary prostate cancer; SNP, single nucleotide polymorphism; PSA, prostate-specific antigen; OR, odds ratio; FMHS, Flint Men's Health Study.

Table 1 *Rare MSR1 germ-line mutations in African-American men with prostate cancer and unaffected controls*

Mutation	Prostate cancer cases (%) (n = 134)	Unaffected men (%) (n = 340)	Fisher's exact test[a]
Ser41Tyr	1 (0.8)	0 (0)	0.278
Asp174Tyr[b]	9 (6.8)	12 (3.6)	0.143
Gly294Glu	0 (0)	0 (0)	
Pro36Ala	9 (7.0)	27 (8.1)	0.847
Arg293X	1 (0.8)	0 (0)	0.278

[a] *P*s based on two-sided test.
[b] Two unrelated cases were homozygous for the Asp174Tyr mutation.

entire sample, with the exception that age at death was used for the 29 controls that died before this date. This age variable was inserted into the models as an independent covariate. All tests were performed at the 5% significance level and using the SAS System (Cary, NC).

Haplotype-based association studies and calculation of the marker–marker linkage disequilibrium measure D′ (7) were performed using the computer program Dandelion (Green, Langefeld, and Lange, unpublished software) following the methodology described in Mohlke *et al.* (8) Briefly, a series of likelihood ratio tests were performed comparing the haplotype frequencies between cases and controls, as estimated by the expectation–maximization algorithm, for two, three, four, and five adjacent marker haplotypes (9). Statistical significance was evaluated using a permutation test based on 1000 random permutations of affection status.

Results and Discussion

The mutation frequencies for five nonsynonymous germ-line mutations are summarized in Table 1. In the first report of rare *MSR1* mutations and prostate cancer, the Asp174Tyr missense change was reported to occur with increased frequency among African-American men with apparent sporadic prostate cancer (4). In our community-based sample, the Asp174Tyr change was identified roughly twice as frequently in men with prostate cancer (6.8%) than in unaffected controls (3.6%; P = 0.14). In addition, 2 of 9 (22.2%) cases were homozygous for the missense change at this allele. Clinicopathologic features for the 9 cases carrying this mutation are summarized in Table 2. Among the 8 patients with available clinical data, 6 (75%) had clinically localized disease at the time of diagnosis. However, 2 cases presented with metastatic disease, with serum PSA levels of 157.8 and 1160 ng/ml, respectively.

Xu *et al.* also reported that the Asp174Tyr missense change cosegregated with prostate cancer in African-American families affected with HPC. Although formal linkage studies were beyond the scope of this case-control study, it is intriguing that at least three (37.5%) carriers of the Asp174Tyr change reported a history of prostate cancer in a first-degree relative, including one man whose family history fulfills the criteria for HPC (two brothers diagnosed with prostate cancer; Ref. 10).

Given that prostate cancer is, in general, a late-onset disease with a long asymptomatic phase, it is also notable that three of the unaffected men carrying the Asp174Tyr mutation had serum PSA levels in excess of 4 ng/ml, and at least two other unaffected carriers have a history of prostate cancer in a first-degree relative (data not shown). Moreover, the mean age of unaffected men with the Asp174Tyr change was 54.2 years, and 6 (50%) of the individuals are ≤50 years of age. This clinical data raises the possibility that, for a number of men, insufficient time may have elapsed to allow phenotypic expression of the underlying genetic variation. Indeed, misclassification of only a few controls may contribute to the lack of statistical significance for the Asp174Tyr mutation in this study sample.

The relative frequencies of the common *MSR1* sequence variants are compared for affected and unaffected men in Table 3. The relative genotype frequencies were similar for cases and controls for each of the common sequence variants with the exception of one nonsynonymous SNP in intron 5 (IVS5-59). For this SNP, heterozygosity (CA *versus* CC) was significantly more common among affected than unaffected men (P = 0.02). For each of the common sequence variants, the allele frequencies and age-adjusted prostate cancer ORs are summarized in Table 4. To estimate the prostate cancer risk

Table 3 *Frequencies of common MSR1 sequence variants in African-American men with prostate cancer and unaffected controls*

SNP and genotype	No. (%) of individuals with Genotype: Control subjects (n = 340)	Case subjects (n = 134)	P[a]
PRO3			
AA	125 (37.5)	54 (41.2)	0.244
AG	166 (49.9)	55 (42.0)	
GG	42 (12.6)	22 (16.8)	
INDEL1			
−/−	125 (37.7)	51 (40.2)	0.228
−/+	166 (50.0)	54 (42.5)	
+/+	41 (12.4)	22 (17.3)	
IVS5–59			
CC	329 (99.4)	122 (96.1)	0.020
CA	2 (0.6)	5 (3.9)	
AA	0 (0)	0 (0)	
P275A			
CC	287 (86.2)	118 (90.1)	0.535
CG	43 (12.9)	12 (9.2)	
GG	3 (0.9)	1 (0.8)	
INDEL7			
−/−	168 (50.9)	71 (56.8)	0.478
−/+	134 (40.6)	43 (34.4)	
+/+	28 (8.5)	11 (8.8)	

[a] χ^2 test.

Table 2 *Clinicopathologic features of nine cases with Asp174Tyr missense mutation*

Case	Genotype	Family history of prostate cancer	Age (years)	Serum PSA at diagnosis (ng/ml)	Clinical stage	Gleason Sum[a]	Type of therapy	Pathologic stage[b]	Metastatic disease
1	Homozygous	No	68.3	20.7	T1cNXMO	7	External radiation		No
2[c]	Homozygous	No	71.7	6.2	T2bNXMO	7	External radiation		No
3	Heterozygous	Yes[d]	59.7	5.2	T1cNXMO	7	Radical prostatectomy	T3aNXMX	No
4	Heterozygous	Yes[e]	63.9	157.8	T3cNXM1	9	Hormonal		Yes
5	Heterozygous	No	64.8	1160.0	T2bNXM1	8	Hormonal chemotherapy		Yes
6	Heterozygous	No	68.5	11.0	T1aNXMO	4	External radiation		No
7	Heterozygous	No	61.4	2.0	T2bNXMO	5	Radical prostatectomy	N/A	No
8	Heterozygous	N/A	57.4	0.6	T2aNXMO	6	Radical prostatectomy	T2aNOMX	No
9[f]	Heterozygous	Yes[g]	50.1	N/A	N/A	N/A	N/A	N/A	N/A

[a] Pathologic Gleason Sum is reported whenever available; otherwise, biopsy Gleason Sum is reported.
[b] For cases undergoing radical prostatectomy.
[c] Additionally carries Ser41Tyr missense mutation.
[d] Brother with prostate cancer.
[e] Two brothers with prostate cancer.
[f] Serum sample provided but epidemiologic questionnaire not completed.
[g] Father with prostate cancer.

Table 4 *Common sequence variant allele frequencies and age-adjusted OR estimates for prostate cancer among African-American cases and controls*

Allele	Allele frequencies (%)		Fisher's exact test for allele (*P*)	Age-adjusted OR (95% confidence interval)
	Control subjects	Case subjects		
PRO3 "G"	37.5	37.8	0.940	0.84[a] (0.55, 1.29)
INDEL1 "+"[b]	37.4	38.6	0.761	0.88[c] (0.57, 1.36)
IVS5–59 "A"	0.3	2.0	0.020	2.90[d] (0.50, 16.79)
P275A "C"	92.6	94.7	0.312	0.72[e] (0.37, 1.40)
INDEL7 "–"[f]	71.2	74.0	0.457	0.78[g] (0.50, 1.19)

[a] Relative odds for AG or GG genotypes *vs.* AA (referent OR = 1.00).
[b] +, the presence of the 15-bp sequence "GAATGCTTTATTGTA."
[c] Relative odds for +/− or +/+ genotypes *vs.* −/− (referent OR = 1.00).
[d] Relative odds for CA or AA genotypes *vs.* CC (referent OR = 1.00).
[e] Relative odds for CG or GG genotypes *vs.* CC (referent OR = 1.00).
[f] −, the absence of the 3-bp sequence "TTA."
[g] Relative odds for +/− or +/+ genotypes *vs.* −/− (referent OR = 1.00).

associated with each sequence variant, we compared prostate cancer risk for one genotype to the combined risk associated with two other genotypes as described previously (3). In this analysis, although the IVS5-59 variant was associated with an increased risk of prostate cancer (OR = 2.9, 95% confidence interval 0.5–16.79), this finding did not reach statistical significance. Haplotype analyses using the five common polymorphisms for all possible combinations of two, three, four, and five adjacent markers revealed no statistically significant findings (minimum $P = 0.2$ obtained for two marker haplotypes defined by IVS5-59 and P275A). Contrary to the findings of Xu *et al.* (3), evidence for marker–marker linkage disequilibrium was observed for all marker pairings, with values of D′ ranging from 1 for marker pairings PRO3—INDEL1, IVS5–59—P275A, IVS5–59—INDEL7 to 0.42 for the marker pairing INDEL1—INDEL7.

Our data provide some additional evidence for a potential link between prostate cancer and germ-line *MSR1* mutations in African-American men. Xu *et al.* (4) reported the presence of the Asp174Tyr change in 6 of 48 African-American men with non-HPC *versus* only 2 of 110 unaffected African-American men. Indeed, when our data are considered in conjunction with these findings, Asp174Tyr mutations are seen in 15 of 182 (8.2%) cases *versus* only 14 of 450 (3.1%) controls ($P < 0.05$). Furthermore, the presence of homozygosity at Asp174Tyr in 2 cases from our sample, one of whom also carried the Ser41Tyr change, raises interesting questions regarding the impact of multiple germ-line mutations on the biology and function of *MSR1*, as well as the coincident effect on prostate cancer risk.

In general, however, our data provide limited support for an association, in African-American men, between prostate cancer and the five common *MSR1* sequence variants. We evaluated each of the common variants that have been reported previously to confer increased prostate cancer risk among men of European descent (3). Statistically significant differences in allele frequencies, among cases and controls, were observed for only one (IVS5-59) of the five sequence variants (Table 3). However, the overall prevalence of this mutation (IVS5-59) in our sample was sufficiently low (2% of cases *versus* 0.3% of controls) that it may be more appropriately classified as a rare mutation rather than a common sequence variant. This discrepancy notwithstanding, after adjustment for age, none of the sequence variants was associated with a significantly increased risk of prostate cancer (Table 4). The results were similar when the control sample was limited to those men who were >50 years of age with screening PSA value(s) < 4 ng/ml (data not shown). Thus, for the common *MSR1* sequence variants, with the possible exception of IVS5-59, our results in a sample of African-American men are inconsistent with those described previously (3).

Xu *et al.* (3) reported previously that each of the common *MSR1* sequence variants, with the exception of INDEL7, was associated with an elevated risk for prostate cancer. However, a recognized limitation of this study was the potential for population stratification, whereby the observed differences in genotype frequencies may partially reflect differing genetic backgrounds among case and control subjects. In contrast, it is more likely that men in our community-based sample come from similar genetic backgrounds, thereby minimizing population stratification and potentially explaining the lack of an association, in our sample, between common *MSR1* sequence variants and prostate cancer risk. Furthermore, it is important to recognize that the study by Xu *et al.* (3) included only men of European descent, whereas our sample was comprised exclusively of African-American men. As a result, it is reasonable that a different conclusion may be reached for African-American men without necessarily compromising the validity and importance of this association in Caucasian men.

There are several limitations to our study. First, we recognize that the relatively small sample size may result in low statistical power for some of our analyses. In addition, selection bias is a potential threat to the validity of all observational studies. Among control subjects in FMHS, <60% of men that completed the initial epidemiological interview participated in the blood draw and clinical examination components of the study. Factors associated with participation in the clinical phases of the project include young age, a family history of prostate cancer, and the presence of urological symptoms (11). Although nonresponse bias is a concern for epidemiological studies of behavioral risk factors, we have no reason to believe that participants and nonparticipants differ systematically with respect to their genetic background.

In conclusion, our analysis of *MSR1* variants in 474 African American men from a community-based study of prostate cancer provides some additional support for an association between rare germ-line *MSR1* mutations and prostate cancer risk. Specifically, we observed that the Asp174Tyr missense mutation is found nearly twice as frequently among prostate cancer cases compared with controls. Although this difference in mutation frequency did not reach statistical significance in our sample, our findings are nonetheless consistent with the hypothesis that this, and potentially other, rare germ-line mutations may mediate prostate cancer risk among African-American men (4). In addition, the IVS5-59 sequence variant may also modify prostate cancer risk among African-American men, and further investigation into the prevalence and functional significance of this change is warranted. We were unable to demonstrate, in African-American men, an association between four other *MSR1* common sequence variants and prostate cancer risk. This study adds to an expanding body of epidemiological evidence in support of the hypothesis that germ-line *MSR1* mutations are risk factors for prostate cancer. Although the evidence from our study is admittedly modest, the public health burden of prostate cancer in the African-American community warrants further investigation of this potential genetic risk factor.

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Polymorphisms in the Prostate Specific Antigen Gene Promoter Do Not Predict Serum Prostate Specific Antigen Levels in African American Men

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ABSTRACT

A major problem with the use of serum PSA in predicting prostate cancer risk is the considerable intra-individual variability of such measurements. Cramer et al. (2003) identified a set of single nucleotide polymorphisms (SNPs) in the upstream regulatory region of the PSA gene that were each associated with increased promoter activity and serum PSA, further suggesting that genotyping these SNPs could be useful in improving the predictive value of PSA screening. In order to replicate this finding, DNA samples from 475 African American men were genotyped for the same SNPs and no association was observed with either serum PSA level or prostate cancer diagnosis.

INTRODUCTION

Prostate cancer is the most common cancer diagnosed among men in the United States with an expected 232,000 new cases in 2005.[1] In addition to increasing age and family history, race is one of the most important recognized risk factors for the disease.[2] African American men have an approximately 1.6 fold greater chance of being diagnosed with prostate cancer compared to Caucasian men and a 2.4 fold greater chance of dying from the disease.[1] There are multiple variables that likely contribute to the racial disparity in both incidence and mortality including genetic, environmental and sociological factors.

Prostate specific antigen (PSA) was first proposed as a serum marker for the early detection of prostate cancer nearly twenty years ago.[3] PSA is a serine protease that is expressed in both normal prostate epithelium as well as prostate cancer. Although serum PSA elevations greater than 4.0 ng/ml are associated with prostate cancer, the positive predictive value (PPV) of a serum PSA value between 4.0 ng/ml and 10 ng/ml is only 20-30%, although the PPV rises to 40-70% for serum PSA values greater than 10ng/ml.[4] A recent analysis of data from the Prostate Cancer Prevention Trial (PCPT) has demonstrated that prostate cancer can also be diagnosed in men with PSA values generally considered to be in the normal range. In the PCPT, approximately 15% of men with normal digital rectal examinations and PSA values consistently under 4.0 ng/ml over a period of 7 years were determined to have prostate cancer on biopsies performed at the end of the study.[5] Clearly, more research needs to be done to improve the use of serum PSA as a screening test for prostate cancer in asymptomatic men.

Transcriptional regulation of the PSA gene is mediated through binding of the androgen receptor to regions of the promoter containing androgen response elements (AREs).[6] In 2003, Cramer et al. reported that specific germline genetic polymorphisms in the promoter region of the PSA gene were associated with higher serum PSA levels among 409 Caucasian male subjects (mean age 63.7 years).[7] They further suggested that these polymorphisms may be useful in refining the recommendations for use of serum PSA levels for prostate cancer screening. Given the importance of prostate cancer in African American men, we set out to explore the potential association between selected functional single nucleotide polymorphisms (SNPs) in the PSA gene with serum PSA levels and prostate cancer risk using samples from the Flint Men's Health Study.

MATERIALS AND METHODS

The Flint Men's Health Study (FMHS) is a community-based study of prostate cancer in African-American men between the ages of 40-79. In 1996, 730 men were recruited to participate in the study from a probability sample residing in the city of Flint and surrounding communities in Genesee County, Michigan. Subjects completed a detailed in-home interview which collected information on sociodemographics, potential risk factors for prostate cancer, and a complete medical history. Subjects were also asked to participate in a clinical examination which included measurement of serum PSA (free and total) and a comprehensive urologic examination. Exam participants were asked to refrain from sexual activity for at least 24 hours prior to the blood draw and all venipuncture was performed before referral for digital rectal exam as both may cause transitory increases in PSA level. Men with an elevated total PSA (=4.0 ng/mL) or an

abnormal digital rectal exam were referred for prostate biopsy. Of the 730 men who completed the initial interview, 379 participated in the clinical exam. Ten subjects were diagnosed with prostate cancer as a consequence of the protocol which resulted in a final control sample of 369 men. Attempts were made to follow the study participants and in the five years after control recruitment, an additional 18 control men were diagnosed with prostate cancer. Four controls with a total PSA level greater than 9.0 ng/mL were also excluded from the analysis following the analysis plan of Cramer et al.[7] A sufficient DNA sample was available for genotyping on 339 of the remaining controls.

Cases were recruited from the same community from 1999 to July, 2002. Men who were between the ages of 40-79 at time of prostate cancer diagnosis (between 1995-2002) were eligible to participate in the study. Cases also completed a detailed epidemiologic interview and provided a blood sample. Medical records were reviewed to extract information related to prostate cancer diagnosis including clinical and pathologic stage, Gleason grade, pre-diagnostic PSA and treatment. A total of 136 cases were ultimately recruited to participate in the study. Informed consent was obtained from all study participants and the research protocol has been approved by the Institutional Review Board of the University of Michigan. For both cases and controls, genomic DNA was isolated from whole blood using the Puregene DNA Purification Kit (Gentra Systems, Minneapolis, MN).

SNP Selection and Genotyping Methods

Three SNPs in the 5' promoter region of the PSA gene were selected: -4643 A/G (rs925013), -5412 T/C (rs2739448), and -5429 T/G (rs2569733). Each of these SNPs were present at greater than 5% frequency in a Caucasian sample and also were shown to

be associated with PSA levels.[7] For the -4643 A/G (rs925013) SNP, Assays on Demand SNP genotyping products (Applied Biosystems, Foster City, CA) were used. These consist of a 20x mixture of PCR primers and dye labeled TaqMan MGB probes designed to genotype a specific SNP within a given sequence. PCR reactions were performed in a 384-well plate format with 2.25 µl of genomic DNA (4 ng/µl), 0.25 µl 20x SNP Genotyping Assay Mix, 1.0 µl 5x Real Time Ready Mastermix (Qbiogene, Montreal, Canada), and 1.5 µl dH_20 for a total reaction volume of 5.0 µl. For the remaining two SNPSs, Assays by Design SNP genotyping products (Applied Biosystems, Foster City, CA) were employed. These assays were custom-designed and produced based upon target sequence submission by using Assay by Design File Builder (Applied Biosystems, Foster City, CA). PCR reactions were performed in a 384-well plate format with 2.375 µl of genomic DNA (4 ng/µl), 0.125 µl 40x SNP Genotyping Assay Mix, 1.0 µl 5x Real Time Ready Mastermix (Qbiogene, Montreal, Canada), and 1.5 µl dH_20 for a total reaction volume of 5.0 µl. Assays were optimized to use a universal thermal cycling protocol with an initial hold at 95 °C for 10 minutes followed by 40 cycles at 92 °C for 15 seconds and a combined annealing extension step of 60 °C for 1 minute. Allelic discrimination was then performed on ABI Prism 7900 HT Sequence Detection System and analyzed using SDS 2.1 software (Applied Biosystems, Foster City, CA). At least 23% of the sample set was duplicated on this platform for verification of results.

Validation of Results

Validation by restriction enzyme digest was performed on 15% of the sample set for the -4643 A/G (rs925013) SNP. PCR primers (5′ CAGGGATTATCTTCAGCACTTACA 3′, 5′ACTGGCCAGCTGGGAATAGAGATA 3′ were designed using Primer Select software

(DNASTAR Inc., Madison, WI) and purchased from Invitrogen Life Technologies (Carlsbad, CA). Each reaction contained 5.0 µl 10x PCR buffer, 1.0 µl 50mM $MgCl_2$, 1.0 µl 10mM dNTPs, 5.0 µl each of the two PCR primers at 5 µM, 2.0 µl genomic DNA at 20 ng/ul, 0.5 µl Platinum Taq Polymerase (Invitrogen Life Technologies), and 30.5 µl ddH_20 for a total reaction volume of 50.0 µl. PCR products were digested using 40.0 µl PCR product, 2.0 µl *NcoI* restriction enzyme, 5.0 µl 10x NE Buffer #4 (New England Biolabs Inc., Beverly, MA) and 3.0 µl ddH_2O for a total reaction volume of 50.0 µl. Samples were placed in a 37 °C water bath and allowed to incubate for at least four hours. Digested products were analyzed on 3% agarose gels.

Validation by direct sequencing was performed on 14% of the sample set for the 5412 T/C (rs2739448), and -5429 T/G (rs2569733) SNPs. For direct sequence analysis, PCR products were purified using Montage PCR Centrifugal Filter Devices (Millipore, Billerica, MA). Cycle sequencing was performed in the forward direction using Big Dye Terminator Chemistries (Applied Biosystems, Foster City, CA). Cycle sequencing reactions were purified using Performa DTR Gel Filtration Cartridges (Edge Biosystems, Gaithersburg, MD) and were sequenced using an ABI 3100 Genetic Analyzer.

Statistical Analysis

For each SNP, the observed genotype distribution was tested for consistency with Hardy-Weinberg equilibrium expectations using Pearson's chi-square test (SAS version 8.2, Cary NC). Lewontin's D′ statistic was calculated to estimate the strength of linkage disequilibrium between all possible pair-wise combinations of SNPs using the computer software GOLD (www.sph.umich.edu/csg/abecasis/GOLD/index.html).[8] PSA levels in our population were not normally distributed after adjustment for age at clinical exam

and genotype. Similar to Cramer et al., a log base-10 (log10) transformation was performed on the PSA levels in order to satisfy distributional assumptions. Analysis of variance was performed to evaluate differences in mean values for log10 total PSA, log10 free PSA and ratio of free to total PSA levels by genotype for each SNP adjusting for age. Because homozygosity for the least prevalent allele was rare in our study population (< 1%), the reported p-values were calculated based on tests comparing the outcome measurements for heterozygotes and homozygotes for the least prevalent allele to homozygotes for the most prevalent allele for each SNP. The least square means for log10 total, log10 free PSA and the ratio of free to total PSA were calculated for each genotype. Logistic regression models were used to test whether each SNP was associated with prostate cancer. Finally, haplotype frequencies, the association between haplotypes and the PSA outcome variables adjusted for age, and the association between haplotypes and prostate cancer were evaluated using the program Haplo.Stat.[9]

RESULTS

The genotype distributions for each of the three SNPs were found to be consistent with expected Hardy-Weinberg equilibrium proportions in both the case and control samples (data not shown). The observed genotype frequencies of the selected PSA promoter SNPs among the FMHS 339 control and 136 case participants are reported in Table 1. Men who were homozygous for the less prevalent allele were rare in this study population. All three SNPs were in near perfect linkage disequilibrium ($p<0.0001$ for all pairs), with pair-wise D' estimates of 0.95, 0.97 and 0.99 for the pairings (-4643, -5412), (-4643, -5429) and (-5412, -5429) respectively.

No significant differences in the frequencies of each of the three SNPs were observed between prostate cancer cases and controls (Table 2). Just one prostate cancer case (0.7%) and one control individual (0.3%) were determined to be homozygous for the least prevalent allele for all three SNPs. Approximately 15 to 16 % of cases and 18 to 19% of controls were heterozygotes for the three SNPs. Consistent with the analyses of PSA levels, statistical significance estimates for the case/control analyses (Table 2) reflect the results of single degree-of-freedom tests comparing the odds of being a case for individuals heterozygous or homozygous for the least prevalent allele to individuals with the most frequent allele. The case/control haplotype analyses failed to detect significant differences in haplotype frequencies between cases and controls (global score statistic = 0.81, p=0.66; data not shown).

Age-specific PSA ranges for control subjects participating in the FMHS have been previously reported.[10] No associations were observed between different genotypes in selected SNPs in the promoter region of the PSA gene with log10 total PSA levels, log10 free PSA levels, or the ratio of free to total PSA (Table 3). Men with the GT genotype at -5429 SNP had similar total PSA levels compared to men with the TT genotype. Likewise, total PSA levels among men with the AG genotype in -5412 SNP were similar to men with the AA genotype. Because SNPs -4643 and -5412 were in almost perfect linkage disequilibrium, serum free and total PSA levels for each genotype in -4643 were nearly identical to those observed at SNP -5412. Of note, the individual who was homozygous for the rare allele at all three SNPs in this study had a higher PSA than the mean values for each of the other genotypes.

In order to examine whether specific combinations of allelic variants were associated with serum PSA levels, we performed haplotype-based association analyses (Table 4). The frequency of haplotypes -5429T/-5412T/4643A and -5429G/-5412C/4643G were approximately 90% and 9%, respectively. No significant differences in log10 total PSA, log10 free PSA or ratio of free to total PSA levels were observed between the three selected haplotypes.

DISCUSSION

Cramer et al.[7] reported that three polymorphisms (-4643 A/G, -5412 T/C and -5429 T/G) located at the far 5' upstream region of the promoter were significantly associated with increased PSA levels using samples from Caucasian asbestos workers participating in a study of asbestos exposure and lung disease. The three SNPs were moderately prevalent in the population, with approximately 21 to 23% of men carrying the variant allele, and the SNPs were shown to be in strong linkage disequilibrium. Further analysis revealed that the -5429T/-5412T/-4643A haplotype, which occurred in 77% of the population, was associated with significantly lower serum PSA levels ($p<0.001$) while the -5429G/-5412C/-4643G haplotype occurring in 20% of the population was associated with higher PSA ($p=0.009$). In our study of a cohort of 339 African American men between the ages of 40 and 79 years with PSA levels less than 9 ng/ml and no known diagnosis of prostate cancer, one haplotype (-5429T/-5412T/-4643A) was detected in 90% of the population and no relationship was detected between any haplotype and PSA level.[7]

A number of studies have examined associations between genetic polymorphisms in the PSA gene, PSA levels and prostate cancer risk. The PSA gene is a member of a family of 15 kallikrien genes clustered on chromosome 19q13.3-13.4.[11] Activity of the gene and expression of PSA is largely mediated through androgen responsive elements located in the proximal promoter and 5' upstream enhancer.[6] Rao et al. initially identified a single SNP within the region, a G/A substitution at -158 bp in the androgen responsive element (ARE I).[12] It was subsequently reported that possession of the A allele was associated with increased serum PSA.[13,14] However, the associations between the ARE I polymorphism with either PSA levels have not been consistent.[15-17] In a study of 518 men, Xu et al. reported no association between polymorphisms in ARE I and PSA levels. The authors suggest that possession of the G allele was associated with increased serum PSA among white men participating in their study, but also note that the highest serum PSA levels among black subjects were observed with the AA genotype. Furthermore, Rao et al. found no difference in serum PSA associated with the ARE I genotype ($p=0.79$). Both studies also examined the relationship between the ARE I genotype and polymorphisms in the androgen receptor gene and with PSA and observed no significant interaction in contrast to other reports.[18]

The A allele at nucleotide position -158 has also been associated with prostate cancer risk in some studies[14,19] but not in all.[17] It is worth noting that some studies report that overall risk appears higher among men with the AA genotype, there are other publications showing that the GG genotype is associated with development of advanced cancer. Xue et al.[18] observed a higher frequency of the GG PSA genotype among men with advanced prostate cancer (evidence of extraprostatic extension, invasion into

surrounding tissue, nodal involvement and/or metastatic disease) (OR = 2.90; 95% CI = 1.24-6.78). A study conducted in China recently reported the GG PSA genotype was associated both with prostate cancer risk overall (OR = 2.27; p=0.008) and also with greater tumor volume and higher pathologic stage.[20] The GG genotype has also been observed more frequently among patients with a Gleason grade of 7 and higher.[19] The inconsistency in reported observations with respect to the -158 SNP in ARE I of the PSA gene are due in part to differences in the racial and ethnic composition of eligible study subjects. For example, the GG genotype at the -158 SNP has is seen in 24% of African Americans men,[13] 29% of Non-Hispanic white men,[13] 61% of Japanese men,[17] and 62% of Chinese men.[20] This fact, coupled with relatively sample size of the study populations, may contribute to the differing results between the ARE I SNP and both serum PSA level as well as prostate cancer diagnosis. Importantly, the -158 SNP has been shown using in vitro assays to have no effect on PSA gene promoter activity.[12]

Using samples from a community-based study of African-American men from the FMHS, we elected to focus on the three PSA promoter SNPs (-4643 A/G, -5412 T/C, and -5429 T/G) upstream from the -158 A/G SNP discussed above. Using transfection assays into the human prostate cancer cell line LNCaP, luciferase reporter constructs containing either the G substitution at -4643 (along with a T at both position -5429 and -5412) or a combination of the C substitution at position -5412 and the G substitution at position -5429 (along with an A at position -4642) each showed more potent promoter activity using transient compared to constructs with the alternative nucleotide at the respective polymorphic site.[7] In addition to the *in vitro* evidence that these variants were functionally important, all three SNPs were shown by Cramer et al. to be in tight linkage

disequilibrium with the -158 ARE I polymorphisms. However, in our study, we were unable to detect evidence that these genetic polymorphisms were associated with either total or free serum PSA levels. The observed frequency of the functional alleles of interest in our study population were approximately half (~10% vs. ~20%) that reported among white men in the Cramer study.

There are distinct differences in the design of each of these studies which might account for some of the inconsistency between findings. The primary aim of the investigation conducted by Cramer and colleagues was to examine the relationship between occupational asbestos exposure and lung disease among asbestos exposed workers. Serum PSA was drawn initially to examine the relationship between asbestos exposure and prostate cancer risk and it is unknown if blood was drawn at the same time of the day for each participant. Evidence suggests that there is significant diurnal variability in PSA levels among men with and without prostate disease and the variation is unpredictable.[21,22] The FMHS protocol required blood draws for all participants between 9 AM and 11 AM to reduce the impact of temporal variability of results. Furthermore, because the participants of Cramer et al. were selected for inclusion into the study based upon their occupation, it is possible that some exposure unique to their occupation, whether asbestos or another substance, might alter their PSA levels. However, it is also possible that the findings represent real racial/ethnic differences in the prevalence and importance of these SNPs in influencing PSA and the utility of these SNPs in predicting PSA and/or prostate cancer risk in Caucasian populations may be greater than in African Americans. Further study is required before any firm conclusions can be drawn with respect to these SNPs in any population.

There are limitations in our study which must be discussed in the context of our findings. First, we have a relatively small sample size which places some limitations on our statistical power to detect small effects due to genotype. Specifically, given our observed genotype frequencies and standard errors in log10 PSA measurements by group, we would have less than 80% power to detect a significant difference (using $\alpha = 0.05$) in PSA level means between genotypes that is smaller than approximately 15%. Second, we did not genotype all SNPs within the PSA gene, therefore other SNPs within the gene may be associated with PSA serum levels in African Americans. We chose to focus on three functionally important SNPs in the promoter region in the PSA gene that have been reported previously as being associated with serum PSA levels. Third, among the control subjects, less than 60% of men who completed the initial epidemiologic survey participated in the blood draw and clinical examination. An analysis of the potential selection bias as a result of response rate in this population revealed that although men who participated in all phases of the study were younger, more likely to have a family history of prostate cancer and were likely to report the presence of urologic symptoms than non-participants, greater participation in the clinical phase of the study did not bias the estimated age-specific reference ranges for total PSA concentrations.[23] Based on these analyses, we have no reason to believe that participants and non-participants differ systematically with respect to their genetic background.

In conclusion, our analysis of 475 African American men with and without prostate cancer does not suggest that SNPs in the 5′ upstream promoter region of the PSA gene influence free or total serum PSA level. Moreover, we did not demonstrate an association between these polymorphisms and prostate cancer risk. The observed

prevalence of the alleles of interest in this community-based study were substantially lower than the prevalence reported in a study of Caucasian men published by Cramer et al. Given the strong linkage disequilibrium between these SNPs as observed in both studies, it will be difficult to establish the independent contribution of each polymorphism. Clearly, additional studies are needed to determine the importance of these SNPs as predictors of serum PSA levels and/or whether these polymorphisms are associated with prostate cancer risk. While enhanced methods for detecting prostate cancer in asymptomatic men are clearly required, it is not clear that PSA promoter polymorphisms will be able to provide much improvement in the current standard of care using serum measurements of PSA.

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Table 1. Frequency of three SNPs in the PSA gene promoter region among African American men.

Location of SNP	Nucleotide Change	% control chromosomes carrying variant (n = 2 x 339)	% case chromosomes carrying variant (n = 2 x 136)
-4643:rs925013 *(NcoI)*	A to G	9.3 (63/678)	8.8 (24/272)
-5412:rs2739448 *(BstUI)*	T to C	9.3 (63/678)	8.8 (24/272)
-5429:rs2569733 *(FokI)*	T to G	9.9 (67/678)	8.5 (23/272)

Table 2. Frequencies of selected SNPs in the promoter region of the PSA gene among 136 men with prostate cancer and 339 healthy control men participating in the FMHS

SNP/Genotype	Prostate Cancer Cases N (%)	Controls* N (%)	OR† (95%CI)
-4643:rs925013 *(NcoI)*			
AA	113 (83.1)	277 (81.7)	
AG	22 (16.2)	61 (18.0)	
GG	1 (0.7)	1 (0.3)	0.91 (0.54-1.55)
-5412:rs2739448 *(BstUI)*			
TT	113 (83.1)	277 (81.7)	
TC	22 (16.2)	61 (18.0)	
CC	1 (0.7)	1 (0.3)	0.54 (0.47-1.55)
-5429:rs256733 *(FokI)*			
TT	114 (83.8)	273 (80.5)	
TG	21 (15.4)	65 (19.2)	
GG	1(0.7)	1 (0.3)	0.80 (0.47-1.36)

* Controls with a serum PSA level of greater than or equal to 9.0 ng/mL were excluded.

† Odds ratio comparing individuals with at least one copy of the rare allele (heterozygotes or homozygotes) compared to individuals who were homozygotes for the more prevalent allele.

Table 3. Least square means, adjusted for age, of log10 total, log10 free and the ratio of free to total PSA by genotype in PSA promoter region SNPs among 339 control participants of FMHS*SNP/Genotype†

	N (%)	Mean Log (Total PSA ng/mL) (SD)	P-value‡	Mean Log (Free PSA ng/mL) (SD)	P-value	Ratio Free:Total PSA (SD)	P-value
-4643:rs925013 *(NcoI)*							
AA	277 (81.7)	-0.039 (0.394)		-0.637 (0.345)		0.272 (0.106)	
AG	61 (18.0)	-0.048 (0.289)		-0.650 (0.230)		0.268 (0.099)	
GG	1 (0.3)	0.613 (N/A)	0.88	-0.268 (N/A)	0.98	0.132 (N/A)	0.65
-5412:rs2739448 *(BstUI)*							
TT	277 (81.7)	-0.040 (0.394)		-0.636 (0.344)		0.273 (0.106)	
TC	61 (18.0)	-0.046 (0.0.294)		-0.652 (0.301)		0.265 (0.099)	
CC	1 (0.3)	0.613 (N/A)	0.86	-0.268 (N/A)	0.90	0.132 (N/A)	0.50
-5429:rs256733 *(FokI)*							
TT	273 (80.5)	-0.037 (0.393)		-0.635 (0.345)		0.272 (0.105)	
TG	65 (19.2)	-0.057 (0.305)		-0.655 (0.303)		0.271 (0.104)	
GG	1 (0.3)	0.613 (N/A)	0.89	-0.268 (N/A)	0.80	0.132 (N/A)	0.84

‡ Age-adjusted comparison between men who are heterozygotes or homozygotes for the least prevalent allele compared to men who are homozygotes for the most prevalent allele.

* Men with a serum PSA level of greater than or equal to 9.0 ng/mL were excluded.

† Nucleotide position relative to the transcription start site.

Table 4. Association between PSA promoter haplotypes and log10 total serum and free PSA levels in 339 controls.

	Allele				Log10 Total PSA		Log10 Free PSA		PSA Ratio	
Haplotype	-5429 T/G SNP	-5412 T/C SNP	-4643 A/G SNP	Estimated Frequency	Score†	P*	Score†	P*	Score†	P*
1	T	T	A	0.900	-.21	0.83	0.11	0.91	0.37	0.72
2	G	C	G	0.091	.37	0.71	-0.10	0.92	-0.71	0.46
3**	G	C	A	0.003	NA	NA	NA	NA	NA	NA
4**	G	T	A	0.003	NA	NA	NA	NA	NA	NA
5**	G	T	G	0.003	NA	NA	NA	NA	NA	NA
Global					0.34	0.84	0.01	0.99		

† HAPLO.SCORE (http://www.mayo.edu/statgen) program statistic.

* Chi-square test of the score test statistic. PSA level adjusted for age.

** Given rarity of haplotype, haplotype not evaluated in statistical tests of association.

A significant p-value and a positive (negative) score for a particular haplotype would have suggested that the haplotype was associated with increased (decreased) levels of the PSA outcome variable.

INS *PstI* Polymorphism and Prostate Cancer Risk in African-American Men: The Flint Men's Health Study

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Abstract

Introduction: African-American men have the highest rate of prostate cancer, and have also been found to have higher levels of serum insulin. It is hypothesized that one of the causes for the higher rate of prostate cancer in African-American men is due to an increased prevalence of the CC genotype of the INS *PstI* polymorphism in the 3'UTR of the INS gene. This genotype is hypothesized to lead to higher levels of serum insulin that subsequently increases the risk of prostate cancer diagnosis. We examined the association between INS PstI genotype and prostate cancer diagnosis in a population-based case-control study of African-American men aged 40-79 using data from the Flint Men's Health Study.

Methods: In 1996, 369 African-American men free of prostate cancer, completed a detailed in-home epidemiologic interview, completed a prostate cancer screening protocol, and had anthropometric measurements and a blood sample taken. 129 African-American men, aged 40-79, residents of Genesee County, and diagnosed with prostate cancer between 1996-2001 were identified using the local cancer registry and completed the interview, had anthropometric measurements and a blood sample taken, and had their hospital and registry records reviewed pertaining to their diagnosis of prostate cancer. Genotype data was collected and analyzed on 124 cases and 342 controls by restriction digests and a sequencing machine. The INS *PstI* genotype was analyzed for its association with prostate cancer diagnosis and severity, and for its association with age of diagnosis of prostate cancer, using multiple variable logistic regression. Other factors including age, BMI, diabetes, and family history were considered in the analyses.

Results: We observed that men with the INS *PstI* CC genotype were more likely to have prostate cancer compared with the TT/TC genotypes (OR=1.63; 95% CI=0.91, 2.91), although this association was not statistically significant (P=0.09). INS *PstI* CC genotype was more highly associated with prostate cancer among men who reported a history of diabetes (OR=2.52, 95% CI 0.84, 7.56). The adjusted odds of INS *PstI* CC genotype was higher for those with T2 cancer (OR=1.50, 95% CI 0.52, 4.28) than for those with T3 cancer (OR=0.96, 95% CI 0.80, 11.54). The adjusted odds of having a higher grade of prostate cancer (Gleason grade =7) are lower for those with the INS *PstI* CC genotype compared to the TT or TC genotype (OR=0.55, 95% CI 0.20-1.55). The CC genotype was associated with a 2.53-fold greater odds of being diagnosed in the 60-69 age category as compared to the 50-59 age category (95% CI 0.67-9.53), suggesting that the CC genotype is associated with a later age of diagnosis. However, the adjusted odds ratio was 1.07 times greater for the CC genotype for being diagnosed in the 70-79 age category compared to the 50-59 age category (95% CI 0.31-3.62), which contradicts this finding.

Conclusion: We observed that INS *PstI* CC genotype was positively associated with prostate cancer diagnosis in this population-based cohort of African-American men aged 40-79 after adjusting for age, BMI, diabetes, and family history. Furthermore, the INS *PstI* genotype was associated with a lower grade and stage of prostate cancer, although these associations were not statistically significant. The INS *PstI* genotype was not associated with a later age of diagnosis as was previously reported. Future longitudinal studies may be necessary that take into account serum insulin levels and that utilize a larger sample size.

Introduction:

Prostate cancer is the most common cancer among American men, and is the second leading cause of cancer deaths in the U.S. There were an estimated 220,900 new prostate cancer cases, and 28,900 deaths due to prostate cancer in 2003[1]. Age, African-American race, and family history are the most well-established risk factors for this disease[2]. African-American men have on average 1.5 times higher incidence of prostate cancer compared to white men, and have 2.5 times higher incidence of distant disease compared to white men[3]. Furthermore, African-Americans have a higher prostate cancer mortality rate than other races, which may be partially due to the fact that they are less likely than whites to be diagnosed with prostate cancer at a localized stage when treatment may be more successful[1]. The likely causes of these racial differences in prostate cancer development and progression have not been clearly elucidated.

Although it is well-recognized that prostate cancer is a hormonally-dependent disease, few studies have been able to document significant associations between serum hormones and prostate cancer risk. This is partially due to the fact that information about normative values of sex-steroid hormones in community-based studies of men is limited. However, it has been well-documented that African American men tend to have higher serum concentrations of insulin[4]. As increased serum insulin levels have been found to be associated with an increased risk of prostate cancer[5], it is plausible to hypothesize that some of the racial difference in incidence of prostate cancer could be explained, in part, by an association between insulin and prostate cancer. One of the hypothesized causes for elevated concentrations of insulin is genetic variation in the insulin gene (INS).

INS is on chromosome 11p15.5 and codes for proinsulin, which is then cleaved by an enzyme and converted to insulin. The variable number of tandem repeats (VNTR) that is adjacent to the 5' INS promoter region is thought to play a part in INS regulation[6]. The class I allele of this VNTR is associated with increased expression of insulin mRNA and insulin levels[7-9]. There are also several noncoding single nucleotide polymorphisms (SNPs) that span the INS gene and its surrounding regions that are in tight linkage disequilibrium with the VNTR such that they constitute two major haplotypes[10-12].

To our knowledge, only one study has examined INS specifically in regards to prostate cancer risk[13]. This hospital-based study analyzed several polymorphisms of the INS gene and the surrounding genes, including the +1127 T/C SNP detected by *PstI* in the 3'-untranslated region (UTR) of the INS gene. The authors report an association between the CC *PstI* genotype and prostate cancer [OR = 1.74 (0.99, 3.05)], and further observed that the association between the CC genotype and prostate cancer was greater for non-diabetic African-Americans [OR = 2.75 (0.88, 8.64)], and increased significantly with age. Finally, the genotype was associated with a low Gleason score and a late age of diagnosis (clinically favorable characteristics). The T allele thus confers a protective effect in this study.

Although this study is the only one to report a potential association between the INS gene and prostate cancer, the study was conducted using a hospital-based sample, which may not be representative of the population at large. In addition, the sample size may have limited the authors from observing statistically significant associations. Finally, the authors did not adjust for important variables such as family history of

prostate cancer and body mass index (BMI) which may confound the association between genotype and prostate cancer risk.

The Flint Men's Health Study (FMHS) is a population-based study of African-American men aged 40-79. The objective of this study was to examine the association between the INS *PstI* single nucleotide polymorphism (SNP) and prostate cancer diagnosis and severity in a population-based cohort of African-American men, adjusted for age, BMI, diabetes, and family history.

Materials and Methods:

The Flint Men's Health Study

The Flint Men's Health Study (FMHS) is a population-based case-control study of prostate cancer in African-American men aged 40-79. Data collection for the study began in 1996 and concluded in 2002. Informed consent was obtained from all study participants, and protocols were approved by the Institutional Review Board at the University of Michigan Medical School.

Controls

A two-stage probability sample of 943 men were selected from households or group dwelling units located in Flint, Michigan or in selected census tracts in neighboring Beecher Township (Genesee County, Michigan). The source population includes all African-American men aged 40-79 living in Genesee County. Men in older age groups were over-sampled to increase the number of eligible subjects for analyses pertaining to

prostate cancer. 817 age and race-eligible men agreed to participate in the study, resulting in a response rate of 87 percent. 87 of the 817 subjects were determined to be ineligible for the study and excluded due to a reported history of prostate cancer or a prior operation on the prostate gland. The 730 remaining subjects completed a detailed in-home epidemiologic interview, which covered information on potential personal and environmental risk factors for prostate cancer; a short family history of cancer; health behaviors such as smoking, drinking and physical activity; general health condition and medical history of chronic illnesses; health care usage; and demographic information.

At the conclusion of the interview, the subjects were asked to undergo a prostate cancer screening protocol, which included providing a blood sample for a serum total prostate-specific antigen (PSA) measurement, and undergoing a comprehensive urological examination which included uroflowmetry, digital rectal examination (DRE), and transrectal ultrasound (TRUS). Participants also completed the self-administered American Urological Association (AUA) Symptom Index and anthropometric measurements were obtained. 379 men completed the protocol. Men with an abnormal DRE and/or elevated total PSA concentration (=4.0 ng/mL) were referred for prostate biopsy. 10 men were diagnosed with biopsy-confirmed prostate cancer and were subsequently excluded from the study, bringing the final number for controls to 369.

Cases

Prostate cancer case recruitment from the same community began in 1999. Cases were identified using the Genesee County Community-Wide Hospital Oncology Program (CHOP) registry, which includes the three hospitals for the county: Hurley Hospital,

Genesys Regional Medical Center and McLaren Regional Medical Center. Case participation in the study required 1) an in-home interview as in the controls; 2) a review of the hospital and registry records for information on stage, Gleason's grade of differentiation, treatment, and pre-diagnosis PSA value; 3) anthropometric measurements; and 4) a blood sample for DNA and freezer storage of serum and plasma. Each hospital's Department of Pathology provided slides and paraffin blocks of prostate tissue samples. An expert in genitourinary pathology at the University of Michigan reviewed the slides and insured storage and preservation of the paraffin blocks and tissues. 129 African-American men aged 40-79 who were residents of Genesee County and who had been diagnosed with prostate cancer between 1995-2002 completed all aspects of case protocol.

Of the 129 eligible cases and 369 controls, an additional 4 cases and 27 controls were excluded due to low DNA concentrations. Thus, 124 cases and 342 controls were included in the final analysis.

Laboratory Methods

Genomic DNA was extracted from whole blood using a commercially available kit (Puregene DNA extraction kit; Gentra Systems, Inc., Research Triangle Park, NC). Primers were designed using PrimerSelect software to amplify a 503-bp region of the INS gene. The forward primer sequence was 5'CGGGGGAAGGAGGTGGGACAT, while the reverse primer sequence was 5'ACAACAGTGCCGGGAAGTGGGG. PCR primers were purchased from Invitrogen (Carlsbad, CA). DNA was amplified by PCR using Promega Taq polymerase with cycling plateaus of 95-56-72 for 45 seconds each

(32 cycles total). Each reaction contained: 29µl ddH_20, 2 µl 25mM MgCl2, 1µ l 10mM dNTPs, 5µl forward primer, 5 µl reverse primer, 2.5µl DNA, .5µl Promega Taq, and 5µl 10X buffer. Amplified DNA was used in a 30µl *PstI* restriction enzyme digest. Each restriction enzyme reaction contained: 3µl 10X NE Buffer 3 (New England Biolabs), .3µl 100X bovine serum albumin (BSA), .15µl *PstI* enzyme, 6.55µl ddH_20, and 20ul amplified DNA. Samples were put in a 37° water bath for at least 4 hours to allow the restriction enzyme to cut the DNA completely. Digested products were size fractionated on 4% agarose gels (NuSieve), and visualized by UV-induced ethidium bromide (Invitrogen) fluorescence and Alpha Imager software. Fragments were sized by comparing to a PBR322 *MspI* ladder. All genotypes were confirmed by a second reader blinded to the previous results, and 18% of genotypes were confirmed by doing an additional restriction digest.

7% of all samples were run through a sequencing machine to confirm genotypes. Before sequencing, PCR products were purified using Microcon PCR centrifugal filter devices (Millipore Corporation, Bedford, MA). PCR products were then cycle sequenced and run through an ABI PRIZM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Only forward sequences were obtained, and all matched previous genotypes.

Measurements

Subjects were considered to be cases if they ever had a confirmed diagnosis for prostate cancer. Controls were those men who reported no prior history of prostate cancer or surgery on the prostate. Controls who were diagnosed with prostate cancer as a

result of the study's screening protocol were subsequently included in the study as cases. INS *PstI* genotypes were dichotomized into two categories: the CC genotype or at least one T allele (TC or TT genotoype) due to the low frequency (n=3) of the homozygous recessive genotype. Subjects were considered to have a positive family history of prostate cancer if a father, son, brother, or uncle had ever been diagnosed with prostate cancer. Age for the cases was defined as the age at which prostate cancer was diagnosed, which was calculated by subtracting the date of birth from the date of diagnosis. Age for the controls was defined as the age at the date of the first interview. Subjects were considered to have diabetes based on the subjects' self-report of physician-diagnosed diabetes. BMI was calculated by dividing weight in kilograms by height in meters squared, using clinical measurements of weight and height and categorized based on the WHO definitions of overweight and obesity: obese (=30), overweight (25=BMI<30), or normal BMI (<25). A pathologist at the University of Michigan Medical Center reviewed information about Gleason grade and stage in the medical records of the cases and analyzed tissue samples to obtain a final measure of grade and stage. Subjects were classified into T1, T2, or T3 stage cancers based on clinical pathology reports. Gleason grade was analyzed as a dichotomous variable of high (=7) or low (=6) grade cancer.

Statistical Analyses

Distributions of the INS *PstI* genotype and other characteristics of the study population were examined overall and by case/control status. Differences in frequencies for categorical variables and means with standard deviations for continuous variables by case/control status were tested using the x2 test and students t-test, respectively. All

analyses were performed using the Statistical Analysis System (SAS, Cary, NC). Two tailed tests were used for all comparisons and p-values of less than 0.05 were considered statistically significant.

Unconditional logistic regression was performed to obtain crude, age-adjusted, and multivariate odds ratios with associated 95% confidence intervals for the association between INS *PstI* genotype and prostate cancer. Multiple variable logistic regression models included variables for INS *PstI* genotype, age, BMI, diabetes, and family history. Stratified odds ratios were calculated to determine whether the association between INS *PstI* genotype and prostate cancer was modified by age, BMI, diabetes, or family history. The Breslow-Day test of homogeneity was calculated to determine whether the odds ratios were significantly different between strata. The Cochran-Armitage trend test was calculated to determine if variables with more than two categories had a linear trend of increasing risk of prostate cancer for each successive category. If age, BMI, diabetes or family history were considered significant effect modifiers in the stratified analyses, the interaction variables were included in the final logistic regression model.

Genotype frequencies and multivariate analyses using unconditional logistic regression were also performed among cases to determine the association between the INS *PstI* genotype and severity of cancer and age of diagnosis.

Results

The distribution of demographic and clinical characteristics of the study population by case/control status are provided in Table 1. Overall, cases were

significantly older than controls, with increasing age significantly associated with prostate cancer (?^{2} trend p<.0001). The odds ratios for the association between INS *PstI* CC genotype increased for each increase in age category. BMI, diabetes, and family history were positively associated with prostate cancer, however these associations were not statistically significant.

Frequency of INS *PstI* genotype by case/control status is provided in Table 2. INS *PstI* CC genotype is positively associated with prostate cancer when compared with the TT/TC genotypes (OR=1.63; 95% CI=0.91, 2.91), although this association was not statistically significant (P=0.09). The CC genotype is most prevalent in both cases and controls, 83.87% and 76.61%, respectively. The TT genotype is rare (n=3). The alleles of the INS *PstI* genotype were found to be in Hardy-Weinberg equilibrium.

Odds ratios for INS *PstI* genotype and prostate cancer stratified by age, BMI, diabetes, and family history are reported in Table 3. INS *PstI* CC genotype was more highly associated with prostate cancer among men who reported a history of diabetes (OR=2.52, 95% CI 0.84, 7.56). However, the association between INS *PstI* genotype and prostate cancer for men with diabetes was not significantly different than the association for men without diabetes (p=0.37). The associations between genotype and prostate cancer did not differ by age, BMI, or family history.

Multiple variable odds ratios for INS *PstI* genotype, age, BMI, diabetes, and family history are reported in Table 4. The INS *PstI* genotype odds ratios were similar in both the age-adjusted (OR=1.68, 95% CI 0.95-2.99) and multiple variable models (OR=1.63, 95% CI 0.91-2.91). BMI was significant in the age-adjusted (OR=1.86, 95%

CI 1.04-3.32) and multiple variable models (OR=1.86, 95% CI 1.03-3.38) for comparing those who are obese with those with a normal BMI, but not for the comparison of those who were overweight with those with a normal BMI. The multiple variable odds ratios for each age category were highly significant and increased with each increasing age category. The age-adjusted and multiple variable odds ratios for family history increased slightly compared to the crude odds ratio, while for diabetes the age-adjusted and multiple variable odds ratios decreased compared to the crude odds ratio.

Table 5 shows the distribution of stage of prostate cancer, percentage of INS *PstI* genotype at each level of stage, and odds ratios of INS *PstI* genotype and stage of prostate cancer at each level of stage, adjusted for age, BMI, diabetes, and family history. Odds ratios compared those in the T2 or T3 category to those in the T1 category. The adjusted odds of INS *PstI* CC genotype was higher for those with T2 cancer (OR=1.50, 95% CI 0.52, 4.28) than for those with T3 cancer (OR=0.96, 95% CI 0.80, 11.54). The Cochran-Armitage test for trend for stage of cancer was not significant (p=0.65). When localized stage cancers (T1 and T2) are combined into one category, the adjusted odds ratio shows that the odds of having regionalized cancer (T3) are lower for those with the INS *PstI* CC genotype compared to the TT or TC genotype (OR=0.76, 95% CI 0.07-8.09). Table 6 shows that the adjusted odds of having a higher grade of prostate cancer (Gleason grade =7) are lower for those with the INS *PstI* CC genotype compared to the TT or TC genotype (OR=0.55, 95% CI 0.20-1.55).

Table 7 shows the distribution of age of diagnosis of cases, percentage of INS *PstI* genotypes in each age category, and odds ratios of INS *PstI* genotype and age of

diagnosis (adjusted for BMI, diabetes, and family history) for each age category. The 50-59 age category was used as the reference category, since the 40-49 group consisted entirely of the CC genotype. The CC genotype was associated with a 2.53-fold greater odds of being diagnosed in the 60-69 age category as compared to the 50-59 age category (95% CI 0.67-9.53), suggesting that the CC genotype is associated with a later age of diagnosis. However, the adjusted odds ratio was 1.07 times greater for the CC genotype for being diagnosed in the 70-79 age category compared to the 50-59 age category (95% CI 0.31-3.62), which contradicts this finding.

Discussion

In this population-based case-control study, we examined the association of the INS *PstI* genotype with prostate cancer diagnosis and severity among African-American men aged 40-79, adjusting for several variables that have been found to be associated with prostate cancer or that may be linked to the insulin gene, including age, BMI, diabetes, and family history of prostate cancer. In this study, we observed that the CC genotype of the INS *PstI* SNP was associated with a 1.63-fold increased odds of prostate cancer diagnosis compared to men with the TC or TT genotypes after adjusting for age, BMI, diabetes, and family history. Age, BMI, diabetes and family history were all found to be positively associated with prostate cancer diagnosis, although only age and BMI were significant in predicting prostate cancer diagnosis in a multivariable model. Furthermore, we observed that INS *PstI* genotype was associated with a lower grade and stage of prostate cancer. Our findings are consistent with previous reports, although the associations were not statistically significant.

High insulin levels have been suggested to be associated with prostate cancer risk, as suggested by previous research, but the mechanisms of this relationship have not been clearly elucidated[5]. It is thought that structural homology of insulin with the insulin-like growth factor (IGF) system, the effect of insulin on prostate epithelial cells, or the effect of insulin on regulating other hormone levels in the body may be responsible for the increase in prostate cancer risk[14]. Variations in the INS gene have been found to be associated with alterations in insulin levels[7-9]. It is possible that the INS *PstI* SNP itself may be associated with altering insulin levels, despite its location in the noncoding 3'UTR of the INS gene. The 3'-UTR of INS suppresses translation and stabilizes the preproinsulin mRNA. It also acts cooperatively with the 5'-UTR and increases glucose-induced proinsulin biosynthesis[15]. It is possible the presence of a T allele at the *PstI* genotype makes the insulin mRNA more stable, thus making it less likely to be translated into the insulin protein. This would lead to lower levels of insulin in the bloodstream, and less opportunity for insulin to act on the prostate and have the potential to contribute to the development of cancer.

Since there is evidence that the INS *PstI* SNP is in strong linkage disequilibrium with other INS SNPs and the VNTR, it may be that *PstI* is just a marker for the actual disease-associated mutation[10-12]. However, due to the fact that the previous study found that INS showed the strongest association with prostate cancer risk, and the strength of association decreased in neighboring genes, this is unlikely. The *PstI* polymorphism of the INS gene is in a region of SNPs that have been found to be associated with a high relative risk of diabetes[12].

The IGF system has been thought to be associated with prostate cancer etiology, and has also been found to be linked to insulin. Insulin has structural homology with IGF-I and IGF-II, and there is homology between the insulin receptor and the IGF-I receptor (IGF-1R) as well[16-17]. These hormones, as well as other elements of the IGF system such as binding proteins and proteases, have been implicated in carcinogenesis due to their role in regulating cell proliferation, differentiation, apoptosis, and transformation[18]. Several studies have found associations between circulating IGF-I concentrations and prostate cancer risk[19-21]. Due to their structural similarity to insulin and their association with prostate cancer risk, it may be beneficial to take IGF hormones into consideration for future analyses.

There is modest, although non-significant evidence that INS *PstI* genotype may be associated with grade and stage of cancer among cases. The odds of having stage T2 cancer is 1.50-fold greater for the CC genotype as compared to the TC or TT genotypes (95%CI 0.52-4.28), whereas the odds of having stage T3 cancer is 0.96 for those with the CC genotype as compared to the TC or TT genotypes (95% CI 0.80-11.54). These are conflicting results, since the direction of the relationship between genotype and stage of cancer changes as stage of cancer increases. However, it may not be clinically meaningful to compare T1 and T2 cancers. When combining stage T1 and T2 into one category of localized cancer, the INS *PstI* CC genotypes were less likely to have T3, or regionalized cancer (OR=0.76, 95% CI 0.07-8.09). However, it should be noted that the sample size was small for T3 cancer (n=4). The INS *PstI* CC genotype was more prevalent in those of lower Gleason grade (=6). The grade of cancer results are similar to

what was reported in the previous study[13], and suggest that the INS *PstI* CC genotype is correlated with a localized cancer.

Using INS *PstI* genotype to predict age of diagnosis of prostate cancer was not consistent in this analysis, however. The CC genotype had the highest odds of being in the 60-69 age category, then the 70-79, then the 50-59. Therefore, the CC genotype was not associated with the latest age of diagnosis as reported in the previous study. When the age categories were divided into three categories to be consistent with the previous study, the CC genotype was still not associated with the latest age of diagnosis (OR=1.39, 95% CI 0.33-5.85 for age = 65 and OR=3.11, 95% CI 0.61-15.97 for age 55-64). The 40-49 age group (n=4) was entirely the CC genotype, which is the opposite of what the previous study suggested. The age distribution was skewed towards the high end in this study (about 70% of cases were diagnosed at age 60-79), which may account for the lack of association detected of genotype and age of diagnosis.

Age and BMI were found to be significant predictors of prostate cancer diagnosis. Since our sample consists of a disproportionate number of obese men (over 30%), the odds ratio associated with BMI in this analysis may be an underestimate of the true effect of BMI, due to the homogeneity of the population with respect to BMI measurement.

Diabetes may be an effect modifier of INS *PstI* genotype and prostate cancer risk, since the genotype/prostate cancer odds ratios varied between those with or without diabetes. Some factor due to diabetes may cause altered insulin levels, such as destruction of pancreatic Islet cells or insulin resistance, which may act in concert with the INS *PstI* genotype to influence the development of prostate cancer. Hsing et al found

that men in the highest tertile of insulin resistance had an increased risk of prostate cancer[22]. In this analysis, 77/90 or 86% of diabetics received treatment for their diabetes. To ensure that those who did receive treatment for diabetes did not differ significantly from those who did not receive treatment or those without diabetes, a Breslow-Day test of homogeneity was performed for genotype/prostate cancer odds ratios in those three groups of subjects. The test was non-significant ($p=0.52$), meaning that treatment for diabetes does not significantly modify the genotype/prostate cancer association. A variable for history of diabetes was considered to be sufficient in this analysis for representing those who have had abnormal insulin levels.

Although this study had many strengths due to the population-based sample selection, consideration of factors such as diabetes and family history of prostate cancer, and inclusion of entirely African-American subjects, this study had some limitations. Since the population was entirely African-American, there was no comparison population to evaluate the risk associated with race. Another limitation is that some controls may have subsequently become cases. Since prostate cancer is a late-onset disease, it is possible that some of the younger controls may not have yet developed cancer. Another limitation is that serum levels of insulin, as well as other related hormones, were not assessed. Due to the cross-sectional nature of the case-control study design, evaluation of temporal trends of hormone levels is not feasible, but even a cross-sectional snapshot of hormone levels can provide insight into the complex nature of how these hormones are associated with prostate cancer risk. There was potential for selection bias, since only half of eligible subjects completed the clinical exam aspect of the FMHS protocol. Participants may have differed from non-participants in INS *PstI* genotype distribution.

Finally, this study was limited in sample size, which is associated with a lower statistical power. One advantage of this analysis is the use of more control subjects than the other study, which does increase the sample size, but the number of case subjects in this study was relatively small. In doing stratified analysis, several of the stratified groups were less than 5, so chi-square values may not have been accurate.

In sum, we observed that INS *PstI* CC genotype was positively associated with prostate cancer diagnosis in this population-based cohort of African-American men aged 40-79 after adjusting for age, BMI, diabetes, and family history. Furthermore, the INS *PstI* genotype was associated with a lower grade and stage of prostate cancer, although these associations were not statistically significant. The INS *PstI* genotype was not associated with a later age of diagnosis as was previously reported. Since there has been only one study to date before this analysis that has looked specifically at INS gene polymorphisms on prostate cancer risk, it is a relatively new discovery and results need to be replicated in other populations, including Caucasian and Asian populations. Future longitudinal studies that can examine the association of INS genotype and prostate cancer diagnosis while considering the impact of levels of endogenous circulating insulin levels over time are necessary to fully elucidate the mechanism of how serum insulin levels and INS genotype interact with each other and impact the prostate.

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Table 1: Sample characteristics by case/control status and odds ratios with 95% confidence intervals for prostate cancer diagnosis				
	Cases (n=124)	**Controls (n=342)**	**Crude Odds Ratio (95% CI)**	
	N (%)	**N (%)**		**$?^2$ trend**
Age				**p<.0001**
70-79	37 (29.84%)	43 (12.57%)	22.80 (7.66, 67.87)	
60-69	51 (41.13%)	86 (25.15%)	15.72 (5.46, 45.21)	
50-59	32 (25.81%)	107 (31.29%)	7.93 (2.71, 23.19)	
40-49	4 (3.23%)	106 (30.99%)	--	
BMI				p=.14
Obese (>30)	38 (31.15%)	95 (27.78%)	1.49 (0.87, 2.55)	
Overweight(25-29.9)	51 (41.80%)	124 (36.26%)	1.53 (0.93, 2.54)	
Normal (<25)	33 (27.05%)	123 (35.96%)	--	
Family history				--
Yes	27 (21.77%)	72 (21.05%)	1.04 (0.63, 1.72)	
No	97 (78.23%)	270 (78.95%)	--	
Diabetes				--
Yes	29 (23.39%)	61 (17.84%)	1.41 (0.85, 2.32)	
No	95 (76.61%)	281 (82.16%)	--	

**Row totals may not add up to the total N due to missing data.

Table 2: Frequency of INS *PstI* genotype by case/control status and odds ratios with 95% confidence intervals for genotype and prostate cancer diagnosis (adjusted for age, BMI, diabetes, and family history)			
	Cases (n=124)	**Controls (n=342)**	**Crude OR (95% CI)***
CC	104 (83.87%)	262 (76.61%)	1.59 (0.93, 2.72)
TC	19 (15.32%)	78 (22.81%)	
TT	1 (0.8%)	2 (0.6%)	--

*Odds ratio compares CC genotype with the TC and TT genotypes combined, due to the small TT sample size (n=3).

Table 3: Stratified odds ratios with 95% confidence intervals of INS *PstI* genotype and prostate cancer diagnosis			
	N	Crude OR (95% CI)	P-value **
Diabetes			
Yes	90	2.52 (0.84, 7.56)	
No	376	1.42 (0.76, 2.64)	P=0.37
BMI			
Obese (=30)	133	1.61 (0.60, 4.34)	
Overweight (25-29.9)	175	1.49 (0.65, 3.41)	P=0.99
Normal (=25)	156	1.65 (0.58, 4.67)	
Family History			
Yes	99	1.44 (0.51, 4.08)	
No	367	1.66 (0.88, 3.13)	P=0.82
Age			
70-79	80	1.75 (0.64, 4.80)	
60-69	137	2.61 (0.91, 7.49)	P=0.35
50-59	139	0.87 (0.33, 2.29)	
40-49	110	*	

*Odds ratio not calculated due to all cases in 40-49 age category (n=4) having the CC genotype.

** Breslow-Day test of homogeneity.

Table 4: Multivariable odds ratios with 95% confidence intervals of INS PstI genotype and prostate cancer risk*	
	Multivariable Odds Ratio (95% CI)
INS *PstI* Genotype	
CC	1.63 (0.91, 2.91)
TT or TC	--
BMI	
Obese	**1.86 (1.03, 3.38)**
Overweight	1.43 (0.84, 2.45)
Normal	--
Diabetes	
Yes	0.90 (0.51, 1.56)
No	--
Family History	
Yes	1.12 (0.64, 1.96)
No	--
Age	
70-79	**34.45 (9.90, 119.91)**
60-69	**21.97 (6.55, 73.63)**
50-59	**11.16 (3.30, 37.76)**
40-49	--

* Variables in model adjusted for all other variables included above.

Table 5: Stage of prostate cancer cases, genotype frequencies, and multivariable odds ratios of INS *PstI* genotype and stage of cancer with 95% confidence intervals (adjusted for age, BMI, diabetes, and family history)				
Stage	N (%)	% CC Genotype	Adjusted OR	Adjusted OR comparing T3 (regionalized) vs. T1 and T2 (localized)
T3	4 (3.51%)	3 (75.00%)	0.96 (0.80, 11.54)	0.76 (0.07, 8.09)
T2	55 (48.25%)	47 (85.45%)	1.50 (0.52, 4.28)	
T1	55 (48.25%)	44 (80.00%)	--	

Chi square trend=.6455

Table 6: Gleason grade of prostate cancer cases, genotype frequencies, and multiple variable odds ratios of INS *PstI* genotype and severity of cancer with 95% confidence intervals (adjusted for age, BMI, diabetes, and family history)

Gleason grade	N (%)	% CC Genotype	Adjusted OR
= 7	63 (53.39%)	50 (79.37%)	0.55 (0.20, 1.55)
= 6	55 (46.61%)	48 (87.27%)	--

Table 7: Frequency of age of diagnosis of cases, genotypes and multivariable odds ratios with 95% confidence intervals of INS *PstI* genotype and age of diagnosis (adjusted for BMI, diabetes, and family history)			
Age at Diagnosis	N (%)	% CC Genotype	Adjusted OR
70-79	37 (29.84%)	29 (78.38%)	1.07 (0.31, 3.62)
60-69	51 (41.13%)	46 (90.20%)	2.53 (0.67, 9.53)
50-59	32 (25.81%)	25 (78.13%)	*
40-49	4 (3.23%)	4 (100.00%)	--

*50-59 was used as the reference group since the 40-49 group is all CC genotype.